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Led Light Photobiomodulation Effect on Wound Healing Combined with Phenytoin in Mice Model

Tarek Elwakil[1], Mahmoud S. Elbasiouny[1], Hatem Elnajar[1], Kawkababdelaziz[2]

[1]Department of Medical Laser Applications, National Institute of Laser Enhanced Sciences, Cairo, Egypt; [2]Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Egypt.

ABSTRACT

Background: Impaired wound healing is a disastrous medical problem associated with chronic diseases and ageing. To accelerate skin regeneration many techniques have been researched laser and LED has been used for these purposes. Topical phenytoin is simple to use, safe, inexpensive and readily available drug and plays a significant role in the rate of healing of wounds.

Objective: We aimed to evaluate irradiation of a LED light 670-nm for wound healing. Using ulcer mice model with and without the combination of phenytoin drug.

Methods: Four groups 24 mice per group received treatment as follows. Group I: Ulcers followed up without any treatment modality.Group II: Ulcers subjected to topical Phenytoin spray twice daily, Group III: Ulcers irradiated with red LED 670 nm at a fluence of 5 J/cm^2. Group IV: Ulcers treated by combined topical Phenytoin spray and red LED670 nm at a fluence of 5 J/cm^2. All treatments started from the 1st day postoperatively and for up to three consecutive weeks or till complete healing of the ulcer.

Results: The results showed the fastest healing in the combined group with more deposition of collagen fibres, larger amounts of granulation tissue, less oedema. The second best treatment was the red LED 670-nm only treated group as mice showed less evident features with fewer collagen fibres deposition.

Conclusion: Red LED 670nm combined with phenytoin is an effective enhancer of wound healing that stimulates the secretion of growth factors in the wound bed and induce many changes during the skin healing process, especially in favouring neo collagen fibres to be better organized and led more deposition of collagen fibres.

Key Words: Wound healing, Biomodulation, Phenytoin, Laser therapy

INTRODUCTION

Many light-based systems had effects on wound healing; these outcomes were noted irrespective of whether a laser, light-emitting diode (LED) or broadband polarized light source used as a Low-level Laser Therapy (LLLT).[1]

This form of therapy is currently being used to treat various conditions based on the principles of photobiomodulation.[2] That influences different biological processes, such as acceleration of wound healing. It is important to know that there is a most favourable light energy needed for wound healing, and higher or lower energy than the favourable value may have no beneficial effect.[3] Wound healing effects are generally observed at fluences between 1 and 10 J/cm^2, while photo inhibitory effects are typically observed at higher fluencies treatment.[4]

Low-level Laser therapy through Photobiomodulation may induce a decisive impact on the course of biological events that take place during wound healing, as it enhances collagen synthesis in the wound area with gradual fibroblastic proliferation and the amount of collagen being synthesized can be particularly affected during tissue regeneration[5] it has been demonstrated that collagen fibres appeared thicker and better organized by a 660 nm wavelength laser application.[6-9]

Several studies have demonstrated that LLLT has a significant influence on a variety of cellular functions. Increased mitochondrial respiration and adenosine triphosphate (ATP) synthesis, cell proliferation, enhancement, and promotion of tissue regeneration following injury. Stimulation of cell proliferation results from an increase in mitochondrial respiration and ATP synthesis. It is assumed that this absorption of

Corresponding Author:
Tarek Elwakil, Department of Medical Laser Applications, National Institute of Laser Enhanced Sciences, Cairo, Egypt;
Email: msaber@niles.edu.eg

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light energy may cause photodissociation of the inhibitory nitric oxide, leading to the enhancement of enzyme activity, increased electron transport, oxygen consumption, mitochondrial respiration, and ATP production.[6] In turn, LLLT, by altering the mitochondrial or cellular redox state, can induce the activation of numerous intracellular signalling pathways and alter the affinity of transcription factors concerned with cellular migration, proliferation, survival, tissue repair, and regeneration.[7]

The basic light interaction behind the effects of LLLT is thought to be through the absorption of a photon by chromophores, in particular, cytochrome c oxidase (CCO), which is part in the respiratory chain positioned within the mitochondria several exogenous factors may interfere with the structural pattern and the number of collagen fibres being deposited during the healingprocess.[8,9]

The possibility of using phenytoin for wound healing was observed in a double-blind, placebo-controlled clinical study involving the use of phenytoin in leg ulcers when the author demonstrated that phenytoin is effective in promoting healing. As it is readily available, safe, inexpensive, and easy to use, It was suggested as a drug for healing ulcers However, no evidence supporting these assumptions of the positive healing effect of phenytoin.[10] The RCTs reported Variable treatment outcomes may be attributed to different doses and forms, different healing assessment tools, topical application of phenytoin powder may improve the rate of healing and is not associated with any serious side effects.[11]

The possible mechanism of action by which phenytoin promotes wound healing has been contributing to an increase in the proliferation of fibroblasts hence increase in the deposition of collagen, increase in Neovascularisation enhanced granulation tissue formation. A decrease in the action collagenase and decrease in bacterial contamination that may be a primary antibacterial effect of phenytoin or if it is due to a secondary effect of phenytoin, such as neo-vascularisation and/or collagenization and there has been at least one study which refutes its beneficial healing effect.[12]

MATERIALS AND METHODS

Animals Model

After the Research Ethics Committee of Cairo University with no. 281472, granted a total of 96 mice between 6 and 9 weeks of age and weighing 18–20 g, were used in this study. The animals were housed one per cage (to prevent cage-mate attacks on wounds) and had access to food and water, free access/*ad-libitum*. The mice were maintained on a 12-hour light/dark cycle under a room temperature of 21 °C.

To produce the surgical wound mice were anaesthetized by an intraperitoneal injection of ketamine–xylazine cocktail (90 mg/kg ketamine and 10 mg/kg xylazine) before wounding the dorsum of the anaesthetized mice were shaved using an electric fur clipper, and the underlying skin was cleaned with sterile 70% isopropanol. The anticipated area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless steel stencil, to ensure comparable wound size in all the mice. A full-thickness excision wound diameter 12mm, along the markings using toothed forceps, a surgical blade and pointed scissors, the wound was left uncovered during the whole period of experiments. Their wounds were cleaned with 0.9% saline once a day in the morning. Animals were monitored daily looking for adverse effects of wounding on their general health.

Light source

The Phototherapy unit used in the study was the LED-based phototherapy system (Photo Therapeutics, Limited, Fazely, Tamworth, UK) which consists of a base unit fitted with interchangeable LED at 670nm (visible red). The illumination performed daily for three weeks, beginning 6h after the induced wound surgery, until 24h before sacrifice. The LED was be used in continuous and directly in non-contact mode with the ulcer surface. The output power of LED was 40mW with fluence 5J /cm2 and Fluence rate (12mW/cm^2) Spot size was defined by the area of the square window in the aluminium foil (1.5×1.5 cm) used to cover the mouse body. Each wound measured approximately 1.2×1.2 cm. Time of irradiation/day was 6.6 min.

Study design

Mice were randomly divided into four groups according to the therapy applied 24 mice per group. The study groups were divided into four groups receives treatment in form of phenytoin or LED or a combination of both as follows. Group I (Control Group): Ulcers were followed up without any treatment modality.Group II (Phenytoin Group): Ulcers was be subjected to topical Phenytoin spray twice daily starting from the 1st day postoperatively and for up to three consecutive weeks or till complete healing of ulcer as denoted by its complete closure. Group III LED (670 nm) Group): Ulcers was be subjected to LED (red spectrum) daily illumination starting after 6 hours postoperatively and for up to three consecutive weeks or till complete healing of ulcer as denoted by its complete closure. The LED treatments were given at a fluence of 5 J/cm^2. Group IV (Combined Phenytoin and LED (Red Spectrum) Group): Ulcers was be subjected to combined topical Phenytoin spray and LED (red spectrum) at the same conditions and parameters as mentioned in groups II and III.

Methods of Assessments

Wound area measurement

Maximum diameters of ulcers were measured daily by Vernier's calibre and ulcer area was be calculated. The relative

change in the surface area of ulcers concerning the initial surface area was determined. Moreover, reepithelization and healthy granulation tissue was grossly evaluated and timings of complete closure of wounds were be assessed for each. Ulcers were photo-documented at different timings of assessments by using a digital camera at the same distance and lighting conditions.

Wound images were acquired every other day using a digital camera. Wound surface area was monitored by capturing the video images of each ulcer area together with a ruler (mm) using the digital camera and downloaded to a computer. The first image of each wound from the different groups was obtained on the day of injury (day 0). The subsequent images were captured on third, fifth, seventh, twelfth and fourteenth-day post-injury. The wound area analysis was performed by Fiji (ImageJ) software and values were expressed in mm^2. Repeated measurements were accurate to within 2%.

Histological Evaluations

Animals were randomly sacrificed at each evaluation time by using an intraperitoneal overdose of ketamine at (3-5-7-10-12-14) day's postoperatively, as well as, after complete healing of ulcers as denoted by the complete ulcer closure. Standardized rectangular specimens were harvested across the wound using a double-blade cutting instrument then preserved in 10 % formalin and then embedded in paraffin. Serial tissue sections of 4-µm thickness were prepared, stained with hematoxylin and eosin (H&E), and observed for histological changes under a light microscope to assess re-epithelialization, epidermis thickness, inflammatory cell infiltration, collagen deposition, and blood vessel proliferation. Staining with Methyl trichrome paraffin sections done to determine the amount of collagen at the wound the most representative findings was be documented by photomicrography.

Immunohistochemical Analysis

Basic fibroblast growth factor vascular endothelial growth factor (VEGF) and tumour necrosis factor were investigated.

Statistical Analysis

Data was be presented as means ± standard deviation (SD). Student's *t*-Test was to be used to determine significant differences between treatment groups. Differences between pairs of means were be analyzed by one–way analysis of variance (ANOVA) test. Values of $P \leq 0.05$ were being considered statistically significant.

RESULTS

Results of Wound area measurements

In the present study, we compared the effect of red wavelength at 635 nm ranges delivered at a constant fluence (5 J/cm^2) and fluence rate (12 mW/cm^2) with and without the combination on of Phenytoin drug that increases angiogenesis and promotes healing to study mice dermal wound healing response variation with wavelength. Wound areas were determined after surgery day after day during the ongoing wound healing process, wound areas decreased in all groups compared to the initial wound area measured after surgery. However, low-level light therapy by a red LED 635 nm combined with Phenytoin markedly influenced this parameter, there was no difference on day 3 post-OP, but on day 7 the wound area was 50% smaller ($p < 0.05$) in the combined LED 635 nm and Phenytoin group compared to not illuminated control to elucidates the effect of light (Figure 1). In contrast, red LED 635 nm light seemed to delay wound closure, and the difference was even greater compared to the Phenytoin group although these findings were not significant.

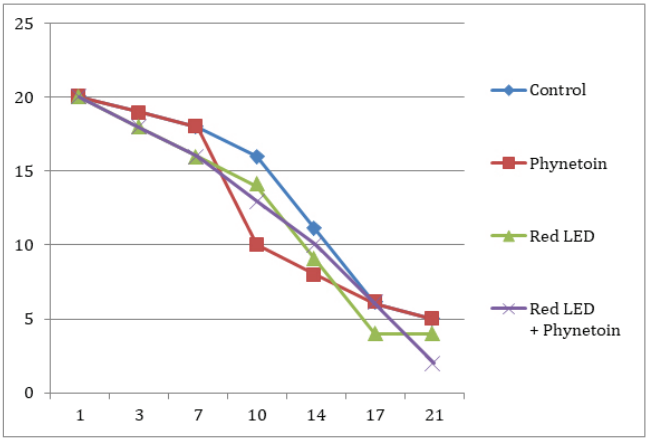

Figure 1: Wound area changes in treatments and control groups.

Results of Morphological wound Assessment

No significant difference could be detected regarding the degree of granulation (Figure 2). However, light affected reepithelialisation as there was a strong trend to enhanced epithelialisation. Wounds treated with different wavelengths exhibited wound contraction from day 1 until observation of day 21 after wounding. However, non-treated control wounds initially expanded on day 1 post-injury, and at day 2, control wounds were the same size as the original size on day 0. From day 4 onwards, the control wound exhibited a gradual reduction in the wound area.

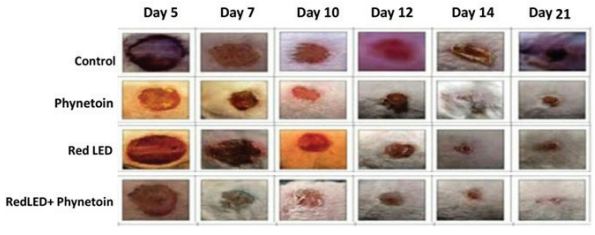

Figure 2: Morphological changes in wounds in the treatment and control group.

Histological Evaluations

Assessments were carried out at (3-7-14-21) days postoperatively were at those times healing/healed ulcers were excised and subjected to multiple cross-sections of H&E-stained sections of wound tissues obtained from the control group and treated groups of mice the evaluations aimed for haemorrhage, epidermis thickness, inflammatory cell infiltration, collagen deposition, and blood vessel proliferation.

Control group: Cross-sections of H&E-stained sections of skin wound from, untreated mice at day 3 showed necrosis associated with oedema and inflammatory infiltrate at day 7 showed necrosis associated with oedema and massive dermal inflammatory infiltrate, at day 14 ill-developed granulation tissue appeared, At 21 days granulation tissue formation with inflammatory cells infiltration and congested newly formed blood capillaries were noticed.

Phenytoin treated group: at day 3 H&E-stained sections showed necrosis, oedema and inflammatory cells infiltration at day 7 it showed necrosis, dermal inflammatory cells infiltration and no haemorrhage, at day 14 showed granulation tissue formation with epithelization well-developed neo angiogenic blood vessels and inflammatory cells infiltration. At day 21 showed epithelization granulation tissue formation collagen fibres.

The Red LED group: at day 3 H&E-stained sections showed necrosis, highly vascularized dermal blood vessel and dermal inflammatory (7days) showing dermal inflammatory infiltrate and congested dermal blood vessel (14 days) showing epithelization and granulation tissue formation

The combined phenytoin and Red LED group: at day 3 H&E-stained sections showed necrosis, dermal inflammatory cells infiltration and haemorrhage at day 7 showed necrosis and massive dermal inflammatory cells infiltration at day 14 showed granulation tissue formation and inflammatory cells infiltration at day 21 showed well-developed granulation tissue formation with few inflammatories infiltrate well-oriented epithelization.

Results of the histopathological comparison scores

On days 3 and 7, the phenytoin and combined phenytoin and red LED groups had significantly higher angiogenesis than the other two groups ($p< 0.05$). Treatment groups were not significantly different among each other of groups 1 and 2 in terms of angiogenesis is. Epithelialization There were no significant differences on days 3 and 21. Epithelialization on day 7 was higher in all the treatment groups compared with the control group ($p< 0.05$). All treatment groups had significantly lower inflammation on day 14 compared with the control group ($p< 0.05$). Red LED light and the combined group had faster epithelialisation than the other treated groups. There were no differences between groups on day 21 shown in Figure 1.

Collagen Analysis

Fibroblastic activity and collagen deposition were determined the amount by Masson trichrome (MTC stain) were not statistically different between treatment groups and the control group Skin of mice from the control group (14 days) showing haphazardly arranged, ill-developed granulation tissue. Notice massive haemorrhage between the granulation tissue Skin of mice from treated with phenytoin (14 days) showing well oriented granulation tissue with collagen fibers deposition Skin of mice from treated with combined phenytoin and led (14 days) showing well oriented, well developed dense stained granulation tissue with collagen fibers deposition Skin of mice from treated with combined phenytoin and led (14 days) showing well oriented, well developed dense stained granulation tissue with collagen fibers deposition Skin of mice from treated with led (Red Spectrum) (14 days) showing well oriented, well developed dense stained granulation tissue with collagen fibers deposition Skin of mice from treated with led (Red Spectrum) (14 days) showing well oriented, well developed dense stained granulation tissue with collagen fibers deposition Skin of mice treated with LED (IR spectrum) (14 days) showing haphazardly arranged, ill-developed granulation tissue kin of mice from treated with combined phenytoin and LED (Red Spectrum) (14 days) showing well oriented, well developed dense stained granulation tissue with collagen fibers deposition (Figure 3).

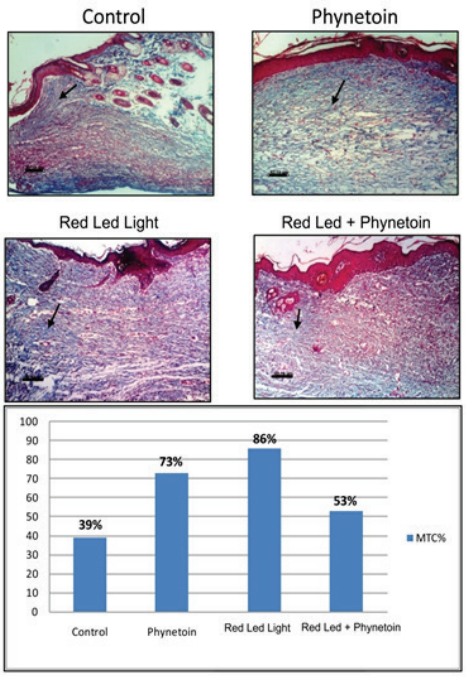

Figure 3: Amount collagen deposition determined by Masson trichrome stain in all the study groups.

Results of the immune histochemical study of the Inflammatory Mediators

Tumour necrosis factor TNF

Immunohistochemical staining of TNF in the skin of control mice showing strong positive expression of TNF in the skin of mice treated with H showing moderate positive expression of TNF skin of mice treated with H showing moderate positive expression of TNF skin of mice treated with HL showing no expression of TNF (negative immunoreaction for TNF). The skin of mice treated with HL showing no expression of TNF (negative immunoreaction for TNF). the skin of mice treated with R showing strong positive expression of TNF skin of mice treated with R showing moderate positive expression of TNF skin of mice treated with RH showing weak positive expression of TNF skin of mice treated with RH showing moderate positive expression of TNF (Figure 4).

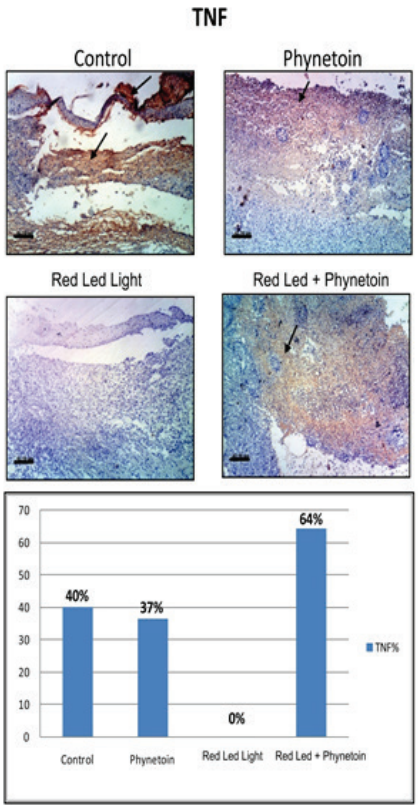

Figure 4: Immunohistochemical stainings of TNF in different study groups.

Vascular endothelial growth factor (VEGF)

Immunohistochemical staining of VEGF in the skin of control mice showing weak positive expression of VEGF Whereas the skin of mice treated with phenytoin showing moderate positive expression of VEGF The skin of mice treated with combined phenytoin and LED (Red spectrum) showing moderate positive expression of VEGF skin of mice treated with combined phenytoin and LED (Red spectrum) showing strong positive expression of VEGF skin of mice treated with LED (Red spectrum) showing strong positive expression of VEGF skin of mice treated with LED (Red spectrum) showing strong positive expression of VEGF skin of mice treated with LED (IR spectrum) showing moderate positive expression of VEGF skin of mice treated with combined phenytoin and LED (IR spectrum) showing strong positive expression of VEGF skin of mice treated with combined phenytoin and LED (IR spectrum) showing strong positive expression of VEGF (Figure 5).

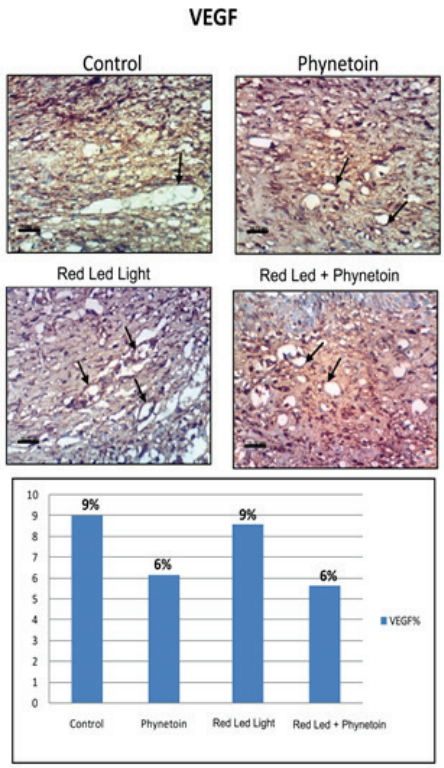

Figure 5: Immuno histochemical staining of VEG in different study groups.

Transforming growth factor (TGF)

Immunohistochemical staining of TGF in the skin of mice from the control group showing no expression of TGF. In the skin of mice treated with phenytoin showing positive expression of TGF in the skin of mice treated with combined phenytoin and LED (Red spectrum) showing positive expression of TGF the skin of mice treated with combined phenytoin and LED (Red spectrum) showing positive expression of TGF skin of mice treated with LED (Red spectrum) showing strong positive expression of TGF skin of mice treated with LED (Red spectrum) showing strong positive expression of TGF the skin of mice treated with LED (IR spectrum) showing moderate positive expression of TGF

skin of mice treated with combined phenytoin and LED (IR spectrum) showing strong positive expression of TGF skin of mice treated with combined phenytoin and LED (IR spectrum) showing strong positive expression of TGF (Figure 6).

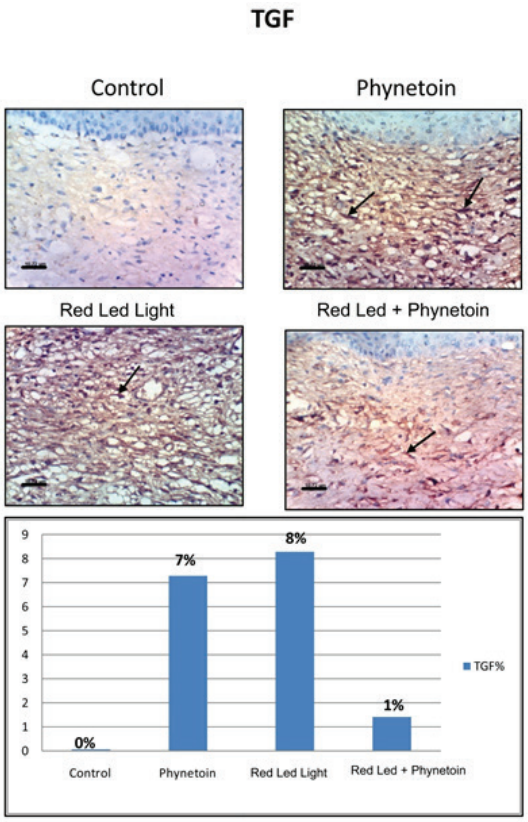

Figure 6: Immunohistochemical staining of TGF in different study groups

DISCUSSION

Various treatment methods are used for the aim of developing wound care products and strategies such as local or systemic agent use, and even surgical treatment.[13-15] The use of light in wound healing experimental studies and clinical applications remains popular among clinicians. Mechanism of Actions of light has been widely investigated to understand the molecular basis and to extend their clinical use.[16] Topical phenytoin had been known helpful for a broad variety of wounds. Clinical studies using topical phenytoin as a treatment suggest that it may be useful for the therapy of both acute and chronic wounds of diverse etiologies.[17] To our knowledge, no other study had tested the combination of phenytoin and LED light therapy for wound healing. The cellular, biochemical, and molecular effects start to build up early in healing wound, as in the cicatricial context.[18] Therefore, the present study aimed to characterize the process of fibrous tissue formation in the later stages of tissue healing. In addition to this aspect, an investigation was conducted to ascertain whether the combination of phenytoin and laser stimulation could affect tissue repair based on the cellular level.[19]

The use of laser therapy for wound healing was initially described as early as 1960. Because of its ability to either stimulate or inhibit tissue responses, the term "biostimulation" was changed to "biomodulation. Several reports have shown that major components of the healing process are affected by several wavelengths, which include: fibroblastic proliferation, the proliferation of keratinocytes, collagen synthesis and deposition, and increased angiogenesis.[19]

Collagen synthesis and other components of the connective tissue are important for the healing process at early stages. However, this process has to be self-controlled to prevent the formation of hypertrophic scars. The positive biomodulation of laser therapy on fibroblastic proliferation and collagen synthesis and deposition are well described in the current literature. In the present investigation, several fibroblasts were observed on irradiated subjects when compared to their controls. These cells were predominantly young and very active in collagen production. Even though the collagen organization observed in the present study suggested that laser therapy influences collagen synthesis but does not significantly affect collagen organization.[20] Our results indicated that non-coherent LED light 670-nm had better stimulatory effects when combined with phenytoin and was superior to it when used alone. On the cellular level, LED light 670-nm modulates fibroblast proliferation, increases collagen bond and synthesis, promotes angiogenesis, improves energy metabolism and produces biological effects during the healing phase.

The collagen deposition varied according to the period and according to the group analysed; it was minor in the great majority of animals in the four groups on the 10th day of examination. Our results harmonize with the findings of previous research that employed wavelengths of 660 and 780 nm and a dosage of 5 J/cm^2 on four points and found a small and moderated number of immature and fragmentary collagen fibres on the 14th day of treatment using optical microscopy.[21]

CONCLUSIONS

The combined phenytoin and LED light to have a positive healing effect on dorsal mice wounds regardless of the radiation dose. The LED light was considered more effective compared to phenytoin in terms of fibroblast proliferation and collagen fibre density. However, further randomized clinical studies are required to determine the effect of the combination of these two modalities of therapy on wound healing in humans.

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